Quantification of Trastuzumab in Rat Plasma using an Improved Immunoaffinity-LC-MS/MS Method

Hybrid Immunoaffinity Solution Featuring SCIEX QTRAP® 6500+ LC-MS/MS System

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Trastuzumab, a monoclonal antibody, is a TNF inhibitor approved for the treatment of early-stage breast cancer that is Human Epidermal growth factor Receptor 2-positive (HER2+) and has spread into the lymph nodes, or is HER2-positive and has not spread into the lymph nodes.[1] Marketed as Herceptin®, trastuzumab has been one of the top-selling pharmaceutical products in the past five years. As such, there is tremendous interest within the clinical research community in analyzing and studying trastuzumab in preclinical samples. However, the procedures are lengthy and complicated for measuring active or free circulating biotherapeutic drug in complex matrices. Active debate on technique selection between LBA and LC-MS has been going on for many years. Key is the ability to achieve the required linearity across the expected pharmacokinetic (PK) sample range.

Here, a universal hybrid LBA/LCMS workflow is introduced combining the advantages of both technologies for protein biotherapeutic PK analysis.

Key Features of SCIEX Hybrid ImmunoAffinity LC-MS/MS Solution

- IonDrive™ system technology on the QTRAP® 6500+ LC-MS/MS system provides:
  - Increased ionization efficiency and ruggedness to provide superior quantitative performance
  - Enhanced dynamic range to cover the appropriate sample concentrations for biologics PK assays without sample dilution
- Bioanalysis by immuno-affinity sample preparation to decrease sample complexity and to achieve desired assay linear dynamic range and reproducibility (CV <15%)
- Simplified sample preparation protocol allows short sample preparation time
- A secondary sample cleanup before introduction to MS analysis is not required

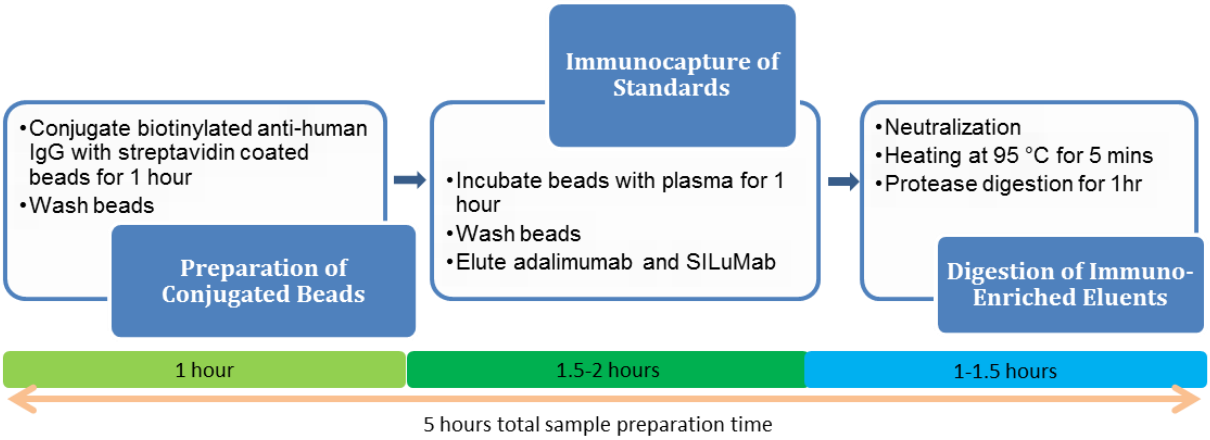

Figure 2. Sample Preparation Workflow. Target protein is captured from plasma using immunoaffinity capture on magnetic beads and matrix is washed away. Protein is then eluted and digested and analyzed by LC-MS/MS.

Methods

Immunocapture of Target Analyte: A streptavidin coated immunoaffinity magnetic bead slurry was aliquoted and washed with PBS Buffer (1x) three times. Biotinylated Goat Anti-Human IgG Antibody (0.5 mg/mL) was added to the beads and incubated at room temperature for 1 hour. The conjugated beads were washed three times and re-suspended in PBS Buffer (1x). SILuMab was prepared as an internal standard. Serial dilution was performed from trastuzumab stock solution to prepare 50 µL of calibration standards by diluting in rat plasma at final concentrations of 10,000, 5,000, 1,000, 500, 100 and 50 ng/mL. To each trastuzumab calibration standard, 100 µL of PBS Buffer (1x) conjugated bead slurry and internal standard were added and the mixtures were incubated at room temperature for 1 hour. The beads were accumulated by magnetic stand and washed sequentially with PBS Buffer (1x) and 10 mM ammonium bicarbonate. The samples were eluted by addition of 0.1% TFA in water and the mixtures were vortexed for 10 min to elute trastuzumab.

Protease Digestion of Immuno-Enriched Eluents: Eluents were transferred to 96 well plate wells and neutralized with Digestion Buffer (1 mM calcium chloride in 500 mM ammonium bicarbonate). The sample plate was placed into a deep well thermo-shaker and incubated at 95 °C for 10 mins with shaking at 1000g. The plate was cooled to room temperature and 1 µg of trypsin was added to each sample. The mixture was incubated for 1 hour at 50 °C. From the mixture 140 µL of the supernatant was transferred to a sample vial.

Chromatography: Separation was accomplished using a Phenomenex® Kinetex®, 2.6 µm, C18 column, 3.0x50 mm at 40°C. Mobile phase A was 0.1% formic acid in water and mobile phase B was 0.1% formic acid in acetonitrile. The needle rinse solution was 33.3% water, 33.2% acetonitrile, 33.3% methanol with 0.2% acetic acid. The flow rate was set at 0.7 ml/min. Injection volume for each analysis was 20µL.The chromatographic gradient is shown in Table 1. Following the gradient, 4 wash steps were executed ramping from 5 to 95% mobile phase B. Overall run time was 10 min.

Table 1. Gradient Profile for Signature Peptide Analysis..

Time (min)	%B
0.7	*5*
0.8	*10*
3.5	*25*
5.0	*40*
5.1	*95*
5.6	*95*
5.7	*5*

Table 1. MRM Transitions for Signature Peptide Analysis.

Name	Q1	Q3	DP	CE	CXP
DTLMIS[R] 2^+y4	*418.2*	*506.2*	*70*	*23*	*15*
DTLMIS[R] 2^+y5	*418.2*	*619.3*	*70*	*22*	*15*
FTISADTSK 2^+y7[1]	*485.2*	*721.3*	*90*	*20*	*15*
FTISADTSK 2^+y6	*485.2*	*608.2*	*90*	*20*	*15*
FTISADTSK 2^+b2	*485.2*	*249.0*	*90*	*20*	*15*

[1]*Most Suitable MRM Transition for Quantitation*

Mass Spectrometry: Signature peptide MRM analysis was performed on a SCIEX QTRAP® 6500+ LC-MS/MS system equipped with IonDrive™ system technology in positive electrospray ionization (ESI) mode using an ion source temperature of 650ºC, IonSpray voltage of 5500, curtain gas pressure of 40 psi, Gas 1 of 65 psi and Gas 2 of 65 psi. The detailed MRM parameters are listed in Table 2.

Results

To select the signature peptides for protein quantitation, the trastuzumab standards were digested in neat solution and analyzed using a TripleTOF® system for peptide mapping analysis. Selection of target peptides follows criteria that consider sequence uniqueness, baseline cleanness in matrix, ionization and fragmentation efficiency, and post-translational modifications. For each peptide, the MRM transition with the highest S/N was selected for quantitation purpose.

With significantly reduced matrix interference provided by immunoaffinity sample preparation and the high sensitivity of the QTRAP 6500+ LC-MS/MS system, the presented assay achieved a LLOQ of 5 ng/mL (Figure 3).

Each sample was analyzed in triplicate. As summarized in Table 3, the assay accuracy is 95-107% and CV% is below 8% for all tested samples. The calibration curve of trastuzumab in rat plasma with SILuMab as internal standard was linear over 4 orders of magnitude (Figure 4) and displayed a regression coefficient (r2) of 0.99502 using a weighting of 1/x2.

Conclusions

A hybrid immunoaffinity-LC-MS/MS method for quantifying trastuzumab in rat plasma is reported. The QTRAP® 6500+ system coupled with ExionLC™ system provides high sensitivity, robustness and broad dynamic range for MRM quantification of peptides. Immunoaffinity sample preparation using magnetic beads significantly eliminates interference from matrix. Combined together, this method provides scientists to quantify trastuzumab at 5 ng/mL in rat plasma.

References

1. www.herceptin.com; the official site from Genentech Inc.

Table 3. Concentration-response linearity data of calibration curve.

Actual Concentration (ng/mL)	Mean Calculated Concentration (ng/mL)	Accuracy (%)	CV (%)
5	*5.27*	*105.38*	*14.03*
10	*9.03*	*90.30*	*9.42*
50	*48.35*	*96.70*	*2.08*
100	*95.46*	*95.45*	*1.39*
500	*496.23*	*99.25*	*3.64*
1000	*1065.31*	*106.53*	*1.21*
5000	*5217.54*	*104.35*	*0.92*
10000	*10854.92*	*108.55*	*0.87*
20000	*18700.07*	*93.50*	*3.88*
50000	*454700.01*	*90.93*	*2.41*

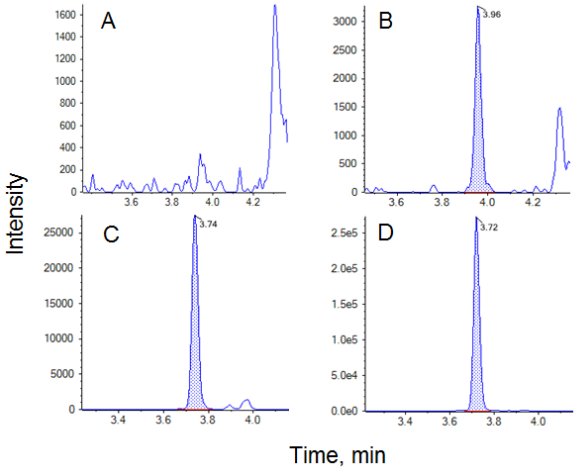

Figure 3. Extracted Ion Chromatograms of Trastuzumab. a) blank; b) 5 ng/mL; c) 50 ng/mL; d) 500 ng/mL.

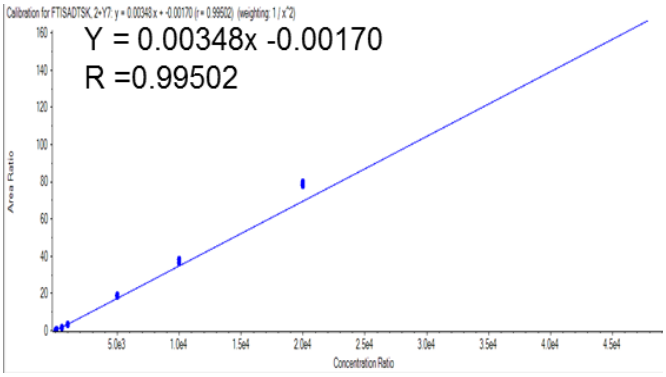

Figure 4. Calibration Curve for Auantitation of Trastuzumab in Rat Plasma (50 ng/mL to 10 000 ng/mL).

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